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GABA-independent GABA$_A$ Receptor Openings Maintain Tonic Currents

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Abstract

Activation of GABA$_A$ receptors (GABA$_A$Rs) produces two forms of inhibition: 'phasic' inhibition generated by the rapid, transient activation of synaptic GABA$_A$Rs by presynaptic GABA release, and tonic inhibition generated by the persistent activation of peri- or extrasynaptic GABA$_A$Rs which can detect extracellular GABA. Such tonic GABA$_A$R-mediated currents are particularly evident in dentate granule cells in which they play a major role in regulating cell excitability. Here we show that in rat dentate granule cells in ex-vivo hippocampal slices, tonic currents are predominantly generated by GABA-independent GABA$_A$ receptor openings. This tonic GABA$_A$R conductance is resistant to the competitive GABA$_A$R antagonist SR95531, which at high concentrations acts as a partial agonist, but can be blocked by an open channel blocker picrotoxin. When slices are perfused with 200 nM GABA, a concentration that is comparable to cerebrospinal fluid concentrations but is twice that measured by us in the hippocampus *in vivo* using zero-net-flux microdialysis, negligible GABA is detected by dentate granule cells. Spontaneously opening GABA$_A$Rs, therefore, maintain dentate granule cell tonic currents in the face of low extracellular GABA concentrations.

Introduction

In addition to fast synaptic GABA$_A$ receptor (GABA$_A$R)-mediated signaling, there is a slower form of signaling resulting from the tonic activation of GABA$_A$Rs (Semyanov et al., 2004; Farrant and Nusser, 2005; Glykys and Mody, 2007a). Tonically active GABA$_A$Rs can have profound effects on neuronal excitability, synaptic plasticity, network oscillations and neurogenesis (Ge et al., 2006; Pavlov et al., 2009; Holter et al., 2010; Mann and Mody, 2010; Martin et al., 2010; Duveau et al., 2011) and have been implicated in neuronal development, information processing, cognition and memory (Semyanov et al., 2004; Farrant and Nusser, 2005; Brickley and Mody, 2012). The conventional view is that tonic currents are mediated by high affinity extrasynaptic GABA$_A$Rs that can detect low

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concentrations of ambient GABA, and therefore the magnitude of tonic currents is regulated by the expression of these receptors and the availability of extracellular GABA (Semyanov et al., 2004; Farrant and Nusser, 2005; Glykys and Mody, 2007a).

The sources and concentration of extracellular GABA ([GABA]$_e$) are, however, still debated. Vesicular release, "leak" through bestrophin channels and reversal of GABA transporters have all been suggested to contribute to the extracellular GABA pool (Attwell et al., 1993; Gaspary et al., 1998; Lee et al., 2010). *In vivo* estimates indicate that [GABA]$_e$ is in the micromolar or submicromolar range (Lerma et al., 1986; Kuntz et al., 2004; Nyitrai et al., 2006). *In vitro* studies have suggested even lower concentrations of GABA, in the nM range; indeed, active uptake maintains GABA at sufficiently low concentrations to prevent tonic GABA$_B$ receptor activation (Isaacson et al., 1993). Thus, tonic currents in hippocampal neurons have often been measured in conditions that artificially raise [GABA]$_e$ by inhibiting GABA uptake or metabolism, or by adding exogenous GABA to the perfusate (Overstreet and Westbrook, 2001; Nusser and Mody, 2002; Stell and Mody, 2002; Holter et al., 2010). When GABA is not increased by these means various magnitudes of tonic currents have been obtained using different GABA$_A$R antagonists (Bai et al., 2001; Mtchedlishvili and Kapur, 2006; Zhan and Nadler, 2009; Mtchedlishvili et al., 2010). Here we report a novel form of tonic inhibition mediated by GABA-independent openings of GABA$_A$Rs, which can explain these discrepancies.

We focus on dentate gyrus granule cells (DGCs), the excitability of which critically depends upon the presence of tonic GABA$_A$R-mediated conductances (Overstreet and Westbrook, 2001; Nusser and Mody, 2002; Stell and Mody, 2002; Coulter and Carlson, 2007; Holter et al., 2010). We demonstrate that under baseline conditions, or when the perfusate contains the same concentration of GABA that is found in cerebrospinal fluid, the major contributors to the tonic current in DGCs are spontaneously opening GABA$_A$Rs. This tonic GABA$_A$R conductance is resistant to the competitive GABA$_A$R antagonist SR95531, but can be blocked by an open channel blocker picrotoxin. On increasing [GABA]$_e$ a SR95531-sensitive component of tonic current emerges. Together these results indicate that the GABA-independent component of tonic current mediated by spontaneously opening GABA$_A$Rs maintains tonic inhibition in the presence of low extracellular GABA concentrations measured *in vivo*.

Materials and Methods

Hippocampal slice preparation

Transverse hippocampal slices (350 μm thick) were used for in vitro electrophysiological recordings. Slices were prepared from 3- to 4-weeks-old male Sprague Dawley rats and $\delta^{-/-}$ knockouts or $\delta^{+/+}$ littermate control mice on a C57B6 background (Herd et al., 2008; Mihalek et al., 1999). Animals were killed by an overdose of isoflurane according to the United Kingdom Animals (Scientific Procedures) Act of 1986. After decapitation, brains were rapidly removed and dissected, and hippocampi were sliced with a Leica VT1200S vibratome in ice-cold sucrose-based solution containing the following (mM): 70 sucrose, 80 NaCl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 22 glucose, equilibrated with 95% O_2 plus 5% CO_2, pH 7.4, 315-330 mOsm. Slices were maintained in continuously oxygenated sucrose-free storage solution at 33°C for 15 min, equilibrated to a room temperature for 15 min and then placed to recover in continuously oxygenated humid interface holding chamber at room temperature for at least 1 hour prior to recording. In experiments with concanamycin, slices were prepared in exactly the same way but before placing them in a holding chamber for the recovery they were incubated for 2 hours in a submerged chamber with continuously oxygenated storage solution and 0.5μM concanamycin. After that slices were placed in an interface holding chamber and allowed to rest for about half an hour prior to recording. After recovering slices were transferred into recording chamber. The perfusion and storage medium contained (mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 22 glucose and was gassed with 95% O_2 and 5% CO_2, pH 7.4; 290–298 mOsm.

In vitro electrophysiology

1. Whole-cell recordings: Visualized patch-clamp recordings from the mature dentate granule cells (R_{in} = 310 ± 50 MΩ; C_m = 48 ± 6 pF) were performed using infrared DIC imaging system. Tonic $GABA_A$R-mediated currents were measured in voltage-clamp mode (V_{hold} = −70 mV) in the presence of ionotropic glutamate receptor blockers DL-APV (50 μM) and NBQX (20 μM), metabotropic glutamate receptors blocker MCPG (250 μM), and $GABA_B$ receptor blocker CGP55845 (1 μM). The intracellular pipette solution contained (mM): 120.5 CsCl, 10 KOH-HEPES, 2 EGTA, 8 NaCl, 5 QX-314 Br^- salt, 2 Mg-ATP, 0.3 Na-GTP, 10 Na-phosphocreatine, pH and osmolarity adjusted to 7.2, 295 mOsm. The tonic $GABA_A$R-mediated current was measured as an outward shift in holding current following application of picrotoxin (100 μM). Changes in root mean square (RMS) noise have also been proposed to reflect changes in tonic $GABA_A$R-mediated conductances and have been used because they are unaffected by current drift. Although RMS noise decreased in experiments in which tonic currents were blocked, this measurement is confounded by the presence of synaptic currents. Moreover, RMS noise is nonlinearly related to current, and can paradoxically decrease when tonic currents increase (Traynelis and Jaramillo, 1998; Glykys and Mody, 2007a). We, therefore, only used RMS noise as a measure in experiments in which we were trying to block the tonic current. Three other $GABA_A$R antagonists, bicuculline methiodide (BMI) (10 μM), gabazine (SR95531) (0.5, 25 and 125 μM), and pentylenetetrazol (PTZ) (1.5 mM) were used in experiments. Gabazine (25 μM) and bicuculline (10 μM) were used at concentrations that would be predicted to give >90% occupancy of $GABA_A$Rs in dentate granule cells (Jones et al., 2001). Gabazine's affinity for $GABA_A$Rs in dentate granule cells is an order of magnitude greater than that of bicuculline (Jones et al., 2001), so that equimolar concentrations of gabazine displace bicuculline from its binding site. Glycine receptors were blocked with 1 μM strychnine, $GABA_C$ receptors – with 50 μM TPMPA. Recordings were performed at 32-34°C. The whole-cell pipette resistance was 4-5.5 MΩ. Series resistance was monitored throughout experiments using a −5mV step command. Cells showing a >20% change in a series resistance, a resistance > 20MΩ, or unstable holding current were rejected.

Acquisition and analysis

Recordings were obtained using a MultiClamp 700B or Axopatch 200B amplifier (Molecular Devices), filtered at 4 kHz, digitized at 10 kHz, and stored on a PC. WinEDR (The Strathclyde Electrophysiology Software) was used for data acquisition and pClamp (Molecular Devices) and OriginPro 8.1 (OriginLab) for off-line analysis.

For analysis of tonic currents mean values of holding current during 200 ms epochs free of synaptic currents were measured every 30 seconds. The amplitude of the tonic current was calculated as the difference between the holding current (ΔI_{hold}) measured 6 minutes before and 10 minutes after the application of an antagonist. With the speed of perfusion in our system of 3.5 ml/min this allowed full equilibration of the solution in the recording chamber. The values of RMS noise were calculated for 200 ms epochs free of synaptic events. The change of RMS noise (Δrms) was calculated as the difference between the values before and after the application of an antagonist.

2. Outside-out and nucleated patch recordings—$GABA_A$R-mediated currents were recorded in the presence of 0.1 μM CGP55845, 100 μM D-APV, 10 μM NBQX, 200 μM S-

MCPG, 1 μM strychnine. The intracellular pipette solution contained (mM): 120.5 CsCl, 10 KOH-HEPES, 10 BAPTA, 8 NaCl, 5 QX-314 Br^- salt, 2 Mg-ATP, 0.3 Na-GTP, pH 7.2, osmolarity 295 mOsm. Patches were pulled from dentate granule cells and recordings were performed in voltage-clamp mode at 33-34°C (V_{hold} = −70 mV) using MultiClamp 700B amplifier. Signals were digitized at 10 kHz. The patch pipette resistance was 5-7 MΩ.

For the rapid solution exchange we used a Θ-glass application pipette with ~200 μm tip diameter attached to the micromanipulator. The position of the pipette was controlled by piezoelectric element (the speed of switch was 50-100 μs). One pipette channel was filled with the bath aCSF solution or bath solution plus 10 μM GABA; another channel had 10 μM GABA, 25 or 125 μM SR95531, 10 μM bicuculline, or 20 μM picrotoxin. Pressure was regulated by a PDES-02DX pneumatic micro ejector (npi electronic GmbH) using compressed nitrogen separately in each of two channels. Solutions with SR95531, bicuculline and picrotoxin were exchanged in a pipette channel (7-12 s) during the exposure of nucleated patch to the bath solution channel (Bennett and Kearns, 2000; Sylantyev et al., 2008).

Analysis of the single-channel recordings

All analyses were performed on stretches of data that were longer than 3 minutes. The opening frequency of $GABA_A$R-mediated channels was calculated as N/Δt, where N is the number of openings and Δt is the time of recording. N was counted using a detection threshold of 1.5 pA more negative than mean baseline and a minimum opening time of 0.2 ms. To calculate single-channel conductance and average open time, we first built all-points amplitude histograms and fitted them with a two-Gaussian function:

$$F=\frac{p_1 e^{-\frac{(n-m_1)^2}{2\sigma_1^2}}}{\sigma_1\sqrt{2\pi}}+\frac{p_2 e^{-\frac{(n-m_2)^2}{2\sigma_2^2}}}{\sigma_2\sqrt{2\pi}},$$

where m_1 and m_2 are the mode values of Gaussians, σ_1 and σ_2 are the standard deviations of corresponding modes, n is the value of electrical current, p_1 and p_2 are the fitting constants. The channel conductance was calculated as G = $(m_2 - m_1)/V_m$.

Newton-Raphson iteration method was used to obtain the value of n_{min}, which is the value of n at the minimum point of the function F in the interval $m_1 < n < m_2$. This was taken as the point of channel closure. Since the signal was digitized at 10 kHz (i.e. at intervals of 0.1 ms), the average open time (in ms) was calculated as follows (the factor of 10 in the denominator is to account for the 0.1ms interval):

$$T=\frac{\sum_{n=n_{min}}^{n_{max}}[F(\mathrm{n})]}{10\times N},$$

where $n_{max} = 2m_2 - n_{min}$

All calculations were performed using the Wolfram Mathematica 6.2 software; the general algorithm of histograms construction and interpretation was adapted from the paper of Bennett and Kearns (Bennett and Kearns, 2000). We compared results obtained using this method against those obtained by averaging all the threshold-detected openings for the data shown in Fig. 3. There was no difference between these two methods (Pearson’s correlation coefficient of 0.992 for open time and 0.979 for conductance).

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Drugs and reagents

GABA receptor antagonists and concanamycin were purchased from Tocris Bioscience. Other reagents were from either Ascent Scientific or Sigma-Aldrich.

In vivo microdialysis

Animals: Male Sprague-Dawley rats (Harlan, Loughborough, UK) were housed three per cage under standard housing conditions (lights on between 05:00 - 19:00, 21 - 22 °C, 50– 60 % relative humidity) with free access to food pellets and drinking water. Animals were handled daily (approximately 5 min/rat) starting one week before surgery and continuing until the day of the insertion of the microdialysis probe. At the time of surgery, rats weighed about 240 g. Surgical procedures were performed under isoflurane (Merial Animal Health Ltd., Harlow, UK) anesthesia. Carprofen (Rimadyl, 4 mg/kg, s.c.; Pfizer, Sandwich, UK) was administered for post-operative pain relief. All procedures were conducted in accordance with the United Kingdom's Animals (Scientific Procedures) Act 1986 and all efforts were taken to minimize animal numbers and suffering.

Surgical and microdialysis procedures: Nine days before the start of the experiment, rats were surgically prepared for microdialysis of the hippocampus by stereotaxic implantation of a guide cannula (MAB 6.14.IC, Microbiotech/se AB, Stockholm, Sweden) essentially as described in detail before (Droste et al., 2008). After surgery, rats were housed individually in Plexiglas cages (length × width × height = 27 × 27 × 35 cm) under similar housing conditions as described above.

Seven days after surgery, a microdialysis probe (polyethersulfone membrane, length 4 mm, 15-kDa cut-off, and outer diameter 0.6 mm, MAB 6.14.4, Microbiotech/se AB) was inserted via the guide cannula into the CA3 – dentate gyrus region of the hippocampus (Linthorst et al., 1994) under short-lasting isoflurane anesthesia (see schematics in Fig. 3*C*). Rats were connected to a liquid swivel and a counterbalance arm (Microbiotech/se AB) allowing free movement in all directions. Fluorethylenepolymer tubing with a dead volume of 1.2 μl per 100 mm length (Microbiotech) was used for all connections. Dead volumes were accounted for during the experiment. Microdialysis probes were perfused with sterile, pyrogen-free Ringer solution (Delta Pharma, Pfüllingen, Germany) at a flow rate of 2 μl/min using a micro-infusion pump (KDS220, KD Scientific, Holliston, MA, USA).

The zero-net-flux experiment was started at 09:00 a.m. on the second day after the insertion of the microdialysis probe. Ten-min samples were collected throughout the complete experiment. After 1 hour of baseline sampling, microdialysis probes were subsequently perfused with increasing concentrations of GABA (10, 20, 40, 80, 160 and 240 nM; Sigma-Aldrich, Gillingham, United Kingdom) dissolved in Ringer solution (reverse microdialysis). Each GABA perfusion lasted 40 min during which four 10-min samples were collected. Microdialysis samples were collected using an automated, refrigerated fraction collector (CMA470, CMA Microdialysis AB, Solna, Sweden) and were stored at −80 °C for later HPLC analysis.

Histology: At the end of the experiment, rats were killed using an overdose of pentobarbital (Euthatal, 200 mg/kg body weight i.p., Merial) and the brains were removed and stored in 4% buffered paraformaldehyde solution. Histological examination was performed as described previously (Linthorst et al., 1994; Droste et al., 2008). Only data from rats with correctly placed microdialysis probes were included in the analyses.

Measurement of GABA: High pressure liquid chromatography with electrochemical detection was used to measure GABA in the microdialysates essentially as described

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previously with small modifications (de Groote and Linthorst, 2007). Briefly, GABA was separated on a TARGA C18 10 cm × 1 mm column (particle size 3 μm; Higgins Analytical, Mountain View, CA, USA) using filtered and degassed mobile phase (18% methanol, 0.1 M NaH_2PO_4, 0.2 mM EDTA, pH 4.72) pumped at 50 μl/min using an Alexys LC-100 pump (Antec Leyden BV, Zoeterwoude, The Netherlands). Standards and samples were injected onto the column using a thermostatically controlled (8 °C) Alexys AS-100 autosampler (Antec Leyden BV). Before injection, 13 μl standard or sample was derivatized with 2 μl of o-phthaldialdehyde (OPA)/sulfite solution (1.6 mM OPA, 0.5% methanol, 1.88 mM Na_2SO_3, 11.25 mM $Na_2B_4O_7$) for 4 minutes to make GABA electrochemically active. Next, 10 μl of the derivatized mixture was injected onto the column and GABA detected using a VT-03 electrochemical flow cell (Antec Leyden BV) set at +850 mV against an Ag/AgCl reference electrode. Both column and detector were housed in a Faraday-shielded oven (DECADE II, Antec Leyden BV) thermostatically controlled at 38 °C. Chromatograms were recorded and analysed using Alexys chromatography software (Antec Leyden BV). The detection limit for GABA at a signal-to-noise ratio of 3:1 was 11-15 fmol per injection on column.

Calculations: Perfusion of each concentration of GABA (C_{in}; nM) was performed for 40 min. However, to ensure that dialysis across the membrane had reached a steady-state, the first 10-min sample was discarded and only the three subsequent samples were used for the calculations described below.

After measurement of the concentration of GABA in the collected samples (C_{out}; nM), the difference between C_{out} and C_{in}, i.e. the net loss or gain of GABA in the dialysate, was calculated (C_{out} - C_{in}; nM) for each concentration of GABA perfused. The baseline condition represented $C_{in} = 0$. Next, for each individual animal, C_{out} - C_{in} was plotted against C_{in} and, after regression analysis (Graphpad 5.0, La Jolla, CA, USA), the concentration of zero-net-flux was determined as the concentration of C_{in} at which $C_{out} - C_{in} = 0$. Next, the mean zero-net-flux concentration (± SEM; n = 5) was calculated. For graphical purposes mean ± SEM values for C_{out} - C_{in} were calculated for each concentration of GABA perfused and were plotted against C_{in} (see Fig. 3*D*).

Statistics—Statistical comparisons were made using paired and unpaired (as indicated in the text/figure legends) Student's t-test, and Wilcoxon signed ranks test (in experiments in Fig. 1*C*). Differences were considered significant when $P < 0.05$. Data are presented in the text and figures as mean ± SEM.

Results

Tonic activation of $GABA_A$Rs in DGCs does not require synaptically released GABA

Since the accumulation of GABA in the extracellular space may result from synaptic release, we asked whether depleting vesicular GABA by incubating hippocampal slices in the vesicular H^+-ATPase inhibitor concanamycin (0.5 μM) would affect tonic currents. We confirmed that concanamycin abolished synaptic currents (Rossi et al., 2003), however it had no significant effect on the magnitude of the tonic current revealed by application of the $GABA_A$R antagonist picrotoxin. Application of picrotoxin produced 11.9 ± 1.5 pA outward shift in the holding current (I_{hold}) in control experiments, and 13.3 ± 3.0 pA shift when slices were pre-incubated in concanamycin (Fig. 1*A*). These results indicate that in acute hippocampal slices, tonic currents in DGCs are not dependent on the concentration of GABA in vesicles or on GABA release into the synapse under baseline conditions. Glycine receptors can contribute to inhibition in the hippocampus and may also be blocked by picrotoxin in a use dependent manner (Danglot et al., 2004; Yang et al., 2007). We tested

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their contribution by adding the glycine receptor antagonist strychnine (1μM) and observed that it had no effect on I_{hold} and did not occlude the effect of picrotoxin (ΔI_{hold} following application of strychnine: −0.8 ± 0.8 pA, n = 5, P = 0.40 compared to control; consecutive application of picrotoxin: 15.6 ± 3.3 pA, n = 4, P = 0.018 compared to strychnine). We further ruled out the potential contribution of $GABA_C$Rs to picrotoxin-induced shift in I_{hold} by applying the $GABA_C$ antagonist TPMPA (50 μM), which also did not affect the amplitude of the picrotoxin-sensitive tonic current (ΔI_{hold} following application of TPMPA: −0.14 ± 1.78 pA, P = 0.9 compared to control; picrotoxin: 11.7 ± 1.7 pA, P = 0.006 compared to TPMPA; n = 4). Thus we conclude that neither glycine receptors, nor $GABA_C$Rs contribute to tonic conductance revealed by picrotoxin.

Tonic currents in DGCs are insensitive to SR95531 under baseline conditions

These results suggest either that $[GABA]_e$ is maintained at a constant concentration despite decreased vesicular GABA release (e.g. by non-vesicular release), and/or that there is another mechanism involved in generating tonic currents that is independent of $[GABA]_e$.

To distinguish between these possibilities, we took advantage of the distinct pharmacologies of different $GABA_A$R antagonists. Picrotoxin is an open channel blocker and acts as a non-competitive $GABA_A$R antagonist that has equivalent efficacy in blocking low affinity synaptic and high affinity extrasynaptic $GABA_A$Rs (Stell and Mody, 2002). In contrast, SR95531 is a competitive $GABA_A$R antagonist, and displaces GABA from its binding site (Hamann et al., 1988). Whilst low concentrations of SR95531 (0.5 μM) partially inhibited and high concentrations (125 μM) totally blocked sIPSCs, there was no significant increase in I_{hold}, which became more negative at high SR95531 concentrations (ΔI_{hold} −6.7 ± 1.6 pA, n = 5, P = 0.014 compared to 0.5 μM SR95531, Fig. 1*B*, Table 1), suggesting a partial agonist effect (this effect was not observed when SR95531 was applied after picrotoxin, ΔI_{hold} −1.2 ± 0.9 pA, n = 3, P = 0.3). Subsequent application of picrotoxin resulted in an outward shift of I_{hold} by 18.9 ± 3.4 pA (P = 0.0049, n = 5), and a decrease in baseline RMS noise by 0.39 ± 0.05 pA (P = 0.0012, n = 5) in the same cells (Fig. 1*B*). This effect of picrotoxin was occluded by prior-application of pentylenetetrazol (Table 1), another noncompetitive $GABA_A$R antagonist (Huang et al., 2001). ΔI_{hold} following application of picrotoxin in the presence of pentylenetetrazol was 0.4 ± 0.7 pA (n = 5).

As previously reported (Semyanov et al., 2003), markedly increasing GABA in the perfusate to 5 μM revealed a SR95531-sensitive component of the tonic current (Fig. 1*C*). Under these conditions, application of a low concentration of SR95531 (0.5 μM) caused a significant outward shift of 29.4 ± 14.4 pA in the holding current (P = 0.016, n = 8) and a significant decrease in RMS noise by 3.0 ± 0.8 pA (P = 0.008, n = 8, Fig. 1*C*). Even in the presence of a high concentration of SR95531 (125 μM) under these conditions, application of picrotoxin caused a further outward shift in holding current by 9.2 ± 2.1 pA (P = 0.031, n = 6), and reduction of RMS noise by 0.51 ± 0.09 pA (P = 0.003, n = 6, Fig. 1*C*).

The lack of efficacy of SR95531 under baseline conditions suggests that only negligible ambient GABA can be detected by dentate granule cells. Alternatively, SR95531 may not bind to the receptors mediating tonic current. The efficacy of SR95531 in expression systems in which α4δ subunit-containing receptors [the main contributor to tonic currents in dentate granule cells (Glykys and Mody, 2007b)] are expressed (Brown et al., 2002) argues against this. Nevertheless, we tested this using another competitive $GABA_A$R antagonist, bicuculline. Application of bicuculline (10 μM) blocked all synaptic activity, induced a small outward shift of holding current by 5.9 ± 0.6 pA (P = 0.00019, n = 6), and decreased baseline RMS noise by 0.72 ± 0.11 pA (P = 0.0014, n = 6; Fig. 1*D*). This result can be explained by bicuculline's inverse agonist activity (Ueno et al., 1997; Bai et al., 2001; McCartney et al., 2007). If SR95531 does not bind to the same receptors as bicuculline then

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it should have no effect in the presence of bicuculline. Conversely if SR95531 does bind then it should displace the bicuculline and paradoxically induce an inward current. The effect of bicuculline was indeed reversed by application of SR95531 (25μM), which activated an inward current of 10.7 ± 1.7 pA (P = 0.0013, n = 6), and increased baseline noise by 0.27 ± 0.06 pA (P = 0.008, n = 6). This was completely blocked by subsequent application of picrotoxin (Fig. 1*D*). These results suggest that all three GABA$_A$R antagonists bind to the same receptors, and that these receptors are not detecting GABA under baseline conditions. Moreover, the lack of effect of SR95531 here indicates that other possible endogenous agonists (such as taurine) are also not mediating the tonic current (Jia et al., 2008).

SR95531-insensitive tonic currents in DGCs are mediated by not only δ subunit-containing GABA$_A$Rs

Tonic GABA$_A$R mediated currents in DGCs are predominantly mediated by α4 and δ subunit-containing receptors (Stell and Mody, 2002; Stell et al., 2003; Caraiscos et al., 2004). We therefore tested whether δ-GABA$_A$Rs contribute to the SR95531-resistant tonic current. We took advantage of knockout mice lacking δ subunits of GABA$_A$Rs. In these mice, in contrast to rats, there was a very small SR95531 sensitive tonic current. However, the majority of the tonic current was SR95531-insenstive (Fig. 2). In δ$^{-/-}$ mice in the presence of 25 μM SR95531, the effect of picrotoxin on holding current was ~60% less than that in wild type mice (littermate controls), implying that δ-GABA$_A$Rs contribute to SR95531-resistant tonic currents (Fig. 2). However, there was a significant SR95531-insensitive tonic current in δ$^{-/-}$ mice, suggesting that other GABA$_A$R subtypes can also contribute to the SR95531-insensitive tonic current in DGCs (Glykys et al., 2008).

Low [GABA]$_e$ is maintained both *in vitro* and *in vivo*

The fact that DGCs display SR95531-insensitive tonic currents suggests that [GABA]$_e$ in *ex vivo* tissue is maintained at concentrations that are not detectable by high affinity GABA$_A$Rs.

We used GABA$_A$R openings in outside-out “sniffer” patches from dentate granule cells (Fig. 3*A,B*) to give a semi-quantitative estimate of [GABA]$_e$ in slices. We first confirmed that channel openings could be recorded in outside-out patches in the presence of 10 μM GABA (channel openings disappeared in the absence of GABA) (Fig. 3*A*). These channels were blocked by picrotoxin and had an opening frequency of 20.1 ± 5.6 Hz, conductance of 39.4 ± 7.2 pS, and average open time of 32.1 ± 7.1 ms (n = 11). We were able to detect GABA$_A$R openings when the patch was 5 μm above the slice (0.55 ± 0.05 Hz, n = 5), indicating that there is sufficient extracellular GABA to bind to GABA$_A$Rs (Fig. 3*B*). These openings did not occur when the patch was moved 300 μm above the slice and recurred on lowering the patch again to the slice (Fig. 3*B*). Consequent application of 200 nM GABA to the perfusate, comparable to that found in cerebrospinal fluid (Glaeser and Hare, 1975), almost doubled the frequency of GABA$_A$R openings recorded above the slice surface. The effect of 200 nM GABA application was even more evident when patches were raised 300 μm above the slice. This indicates that [GABA]$_e$ < 200nM in the slice (Fig. 3*B*), and is consistent with the predicted GABA transporter equilibrium [GABA]$_e$ of approximately 100 nM (Wu et al., 2007). We further confirmed that similarly low [GABA]$_e$ is maintained *in vivo* by using the zero-net-flux microdialysis method. Microdialysis probes were inserted into the CA3-dentate gyrus region via guide cannula, and *in vivo* [GABA]$_e$ was calculated to be 92 ± 10 nM (n = 5; Fig. 3*C, D*).

Since an increase in GABA$_A$R openings occurs as the patch approaches the surface, yet there is no evidence of significant GABA-medicated tonic currents, it is likely that the activity of GABA transporters prevents extrasynaptic $GABA_A$ receptors from being exposed to even such low concentrations under baseline conditions. When GABA in the perfusate was increased to 200 nM, comparable to that found in cerebrospinal fluid (Glaeser and Hare, 1975), I_{hold} did not change significantly and SR95531 (25 μM) had no significant effect on I_{hold} (ΔI_{hold} following consequent application of GABA: −1.21 ± 2.03 pA, P = 0.6; SR95531: 1.7 ± 1.4 pA, P = 0.29; n = 5), indicating that there is no increase in the GABA detected by extrasynaptic $GABA_A$Rs.

Spontaneously opening $GABA_A$Rs in DGCs

The evidence thus far implies that tonic inhibition in DGCs *in situ* is predominantly GABA-independent. Can tonic currents be mediated by spontaneously opening $GABA_A$Rs? Such receptors have been reported in excised patch and cell-attached recordings from hippocampal neurons (Birnir et al., 2000a), but not in the DGCs (Birnir et al., 1994). Failure to detect spontaneously opening $GABA_A$Rs may be due to the small area sampled using outside-out patches, and/or the dependence of such openings upon the internal neuronal environment. Indeed, we noted that in very few excised patches (less than 10%) could we detect infrequent channel openings (less than one every 20 seconds). We therefore performed recordings from nucleated patches (i.e., whole-cell excisions containing intact nuclei). In this preparation spontaneous $GABA_A$R openings were recorded in the majority of patches (Fig. 4). These openings had a conductance (37.4 ± 6.1 pS) similar to that observed in excised outside-out patches with applied GABA (39 ± 7.2 pS; Fig. 3*A*), and comparable to that determined in other studies (Bright et al., 2011). As expected spontaneous openings were not affected by SR95531 (25 μM), but were fully blocked by picrotoxin (Fig. 4*A-D*). Since a high concentration of SR 95521 (125 μM) had acted as a partial agonist (see Fig. 1), we tested the effect of SR 95521 (125 μM) on spontaneous openings in nucleated patches and, as predicted, found that it increased the frequency of openings (Fig. 4*E*). Addition of 10 μM GABA to the nucleated patches revealed SR95531-sensitive channels with the same conductance (Fig. 5).

Discussion

Our results indicate that GABA-independent openings of $GABA_A$Rs are the major contributor to tonic currents in dentate granule cells *ex vivo*, even when GABA concentrations in the perfusate are increased to levels comparable to those measured *in vivo*.

We took advantage and confirmed the different pharmacologies of $GABA_A$R antagonists (Table 1), and have shown that *in situ* DGC tonic currents are resistant to SR95531, indicating that they are not being generated by GABA binding to the receptors. This is consistent with similar observations in cultured neurons, other brain areas and cell types (Birnir et al., 2000b; McCartney et al., 2007). Importantly, SR95531 has a paradoxical effect of inhibiting the action of the inverse agonist bicuculline. This result suggests that SR95531 is displacing bicuculline from the receptors and consequently that the lack of effect of SR95531 is not due to a failure to bind to the receptors that are mediating the tonic current. At high concentrations (125 μM), we also found that SR95531 can act as a partial agonist. A partial agonist effect of SR95531 (100 μM) and bicuculline (1 mM) has been described previously at $GABA_A$Rs in which there is a point mutation in the γ subunit (Ueno et al., 1997). We did not test bicucline at this concentration and so cannot exclude a partial agonist effect of very high concentrations of bicuculline at extrasynaptic receptors.

This lack of GABA binding explains why inhibiting vesicular GABA release in our studies has no effect on the tonic current. The low concentration of GABA despite vesicular release is almost certainly due to efficient GABA uptake, as when GABA transporters are blocked, tonic currents become dependent upon vesicular GABA release (Glykys and Mody, 2007b).

The effect of decreasing vesicular release has variable results depending upon the nature of the tissue studied, suggesting that GABA concentrations and the consequent detection of extracellular GABA by $GABA_A$Rs varies between brain regions. For example, in thalamocortical neurons, 50 μM SR95531 has been demonstrated to reveal robust tonic $GABA_A$R-mediated currents (Cope et al., 2005). There may even be local inhomogeneities of GABA concentrations due to regional differences in the distribution of GABA transporters (Semyanov et al., 2003). Indirect evidence of this in the hippocampus is supplied by the observation that tonic currents are reduced in CA1 interneurons, but not in pyramidal cells of GAD65 deficient mice (Song et al., 2011). However, whether the heterogeneity of extracellular GABA concentrations observed *in vitro* occurs *in vivo* is unclear. Spontaneous openings of $GABA_A$Rs have been previously described in some preparations (e.g. in isolated hippocampal pyramidal cells and in outside-out patches from pyramidal neurons, but not DGCs). However, the role for these spontaneous openings, and whether they can contribute to $GABA_A$R-mediated signaling *in situ* has remained unclear (Macdonald et al., 1989; Birnir et al., 2000b). Here, we recorded from nucleated patches to show directly that spontaneously opening receptors are present in dentate granule cells. As predicted from other studies (McCartney et al., 2007), they are not affected by the competitive $GABA_A$R antagonist SR95531 and are completely blocked by the open channel blocker picrotoxin. Lack of evidence of such openings in outside-out patches from dentate granule cells in previous studies may be related both to the small area of membrane and also to the effects of the internal cellular milieu on $GABA_A$R channel gating.

A vexed question is how GABA in a slice relates to the *in vivo* situation. Direct measurements of GABA in cerebrospinal fluid have revealed concentrations of the order of 200 nM (Glaeser and Hare, 1975). Using the zero-net-flux microdialysis method, we have estimated the concentration in the extracellular fluid to be even lower. This is somewhat at odds with an earlier study which estimated the GABA concentration in the extracellular fluid of the hippocampus to be 800 nM (Lerma et al., 1986). This measurement however was confounded by the complex method used to retrieve absolute concentrations from dialysate concentrations and reliance on estimates of diffusion across the dialysis membrane. It should also be noted that these previous estimates were done in rats under urethane anesthesia, and sampling was performed one hour after probe implantation, when the blood-brain-barrier could still be disrupted. The microdialysis method averages over a large area and so we cannot exclude the possibility that the regional GABA concentrations may differ and, in particular, that the concentration detected by neurons may be greater than this depending upon the balance of local release and uptake; however, this was not observed by us (and others) using the “sniffer patch” technique. Indeed, 100 nM is close to the EC20 for extrasynaptic δ-subunit containing $GABA_A$Rs (Bright et al., 2011), and so the absence of a detectable GABA-mediated current under baseline conditions or when GABA in the perfusate is increased to 200 nM indicates that the GABA detected by neurons is less than that present in the extracellular space, perhaps due to efficient local GABA uptake. These results strongly argue against the widespread addition of high GABA concentrations (usually 5 μM) to the perfusate during *ex vivo* experiments. Indeed, these concentrations of GABA are fifty fold greater than those we detect *in vivo*.

Our findings have several important implications. First, our results indicate that studies that use SR95531 to measure tonic currents may significantly underestimate them. The results may also explain discrepancies in the measurement of tonic currents in DGCs, which in one study (and in contrast to others) was proposed not to be present in control tissue because of the lack of effect of SR95531 (Zhan and Nadler, 2009). Furthermore the widespread use of SR95531 and on occasions high concentrations of SR95531, which could have a partial agonist effect, may significantly underestimate the magnitude of tonic currents present. Even when GABA is gating the receptor, displacement by SR99531 would not prevent

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spontaneous openings, and may therefore not accurately measure the tonic current. Moreover, our results indicate that there is always a tonic current present in dentate granule cells even when extracellular GABA concentrations are low (as they are most of the time). The importance of maintaining such inhibitory tone in neurons is further underscored by the observation that knocking out receptors generating tonic current in cerebellar granule cells leads to an upregulation of a two pore potassium channel in order to maintain the conductance (Brickley et al., 2001).

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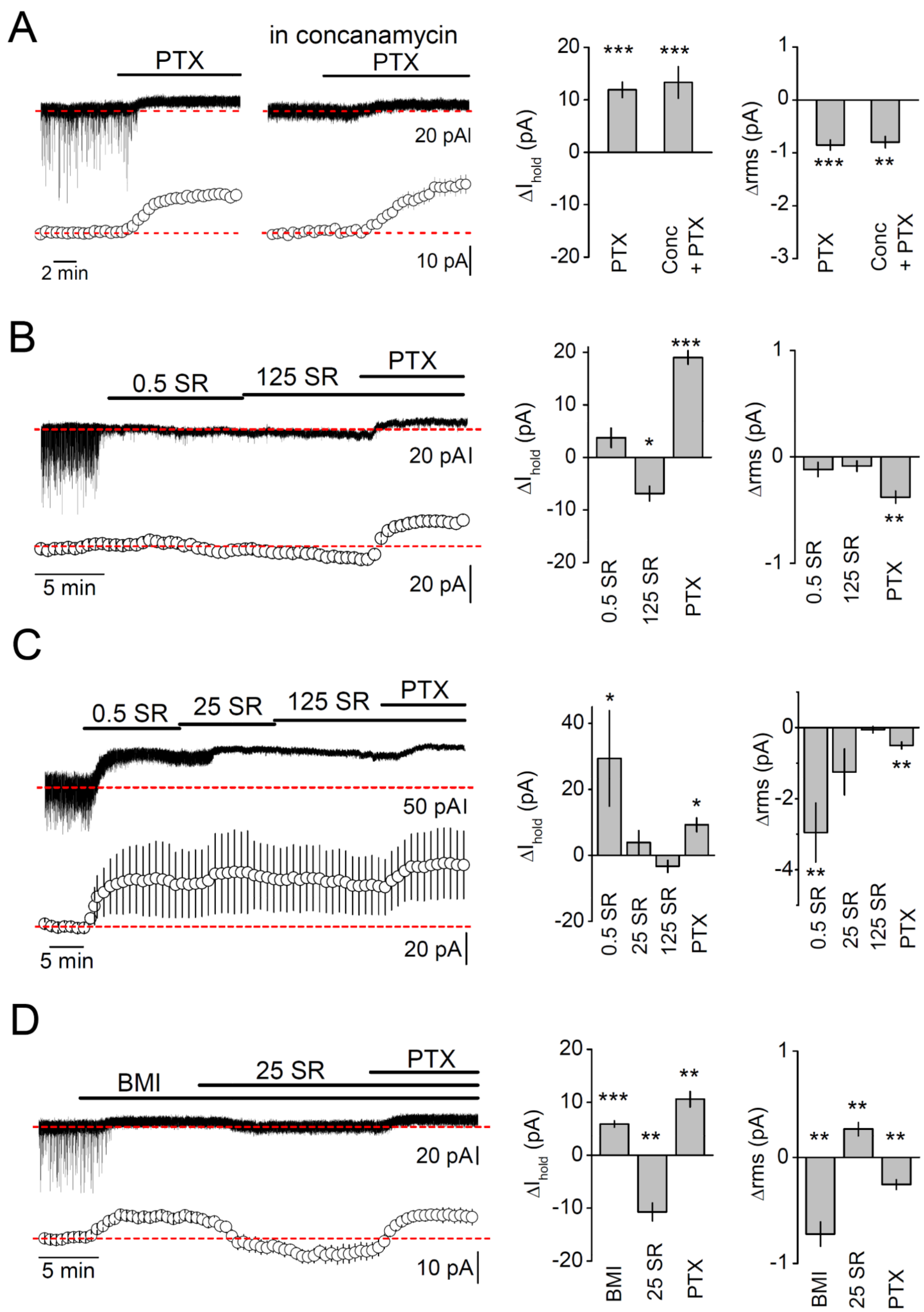

Figure 1.

Pharmacology of the $GABA_AR$-mediated tonic currents in DGCs. ***A***, Concanamycin (0.5 μM) blocks exocytosis but does not affect I_{tonic}. No significant difference between the change in I_{hold} or RMS noise in control (*left trace*, n = 9) and concanamycin treated slices (*right trace*, n = 6) upon application of picrotoxin (PTX, 100 μM). ***B***, I_{tonic} in DGCs is insensitive to SR95531 and abolished by picrotoxin. SR95531 (0.5μM) partially inhibited, whilst high concentrations (125 μM) completely abolished sIPSCs. Picrotoxin induced an outward shift in I_{hold} (P = 0.0049; n = 5), and decreased RMS noise (P = 0.0012; n = 5). ***C***, In the presence of GABA (5 μM), application of SR95531 (0.5 μM) reduced sIPSCs, induced an outward shift in I_{hold} (P = 0.016) and decreased RMS noise (P = 0.008; n = 8).

SR95531 (25 μM) abolished sIPSCs, induced a further non-significant shift in I_{hold} (P = 0.44) and RMS noise (P = 0.06; n = 5). SR95531 (125 μM) affected neither I_{hold}, nor RMS noise. Consequent application of picrotoxin produced outward shift in I_{hold} and reduced RMS noise (P = 0.031 and P = 0.003 respectively; n = 6). ***D***, Bicuculline (BMI) (10 μM) blocked all synaptic activity, induced outward shift in I_{hold} and reduced RMS noise (P = 0.00019 and P = 0.0014 respectively; n = 6). SR95531 (25μM) resulted in inward current and an increase in RMS noise (P = 0.0013 and P = 0.008 respectively; n = 6), which were reversed by picrotoxin (100 μM) (P = 0.0019 and P = 0.0048 respectively; n = 5). Statistical comparisons were made using paired Student's t-test in ***A***, ***B*** and ***D*** and Wilcoxon signed ranks test in ***C***. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ΔI_{hold} and ΔRMS noise values represent changes from the previous drug application.

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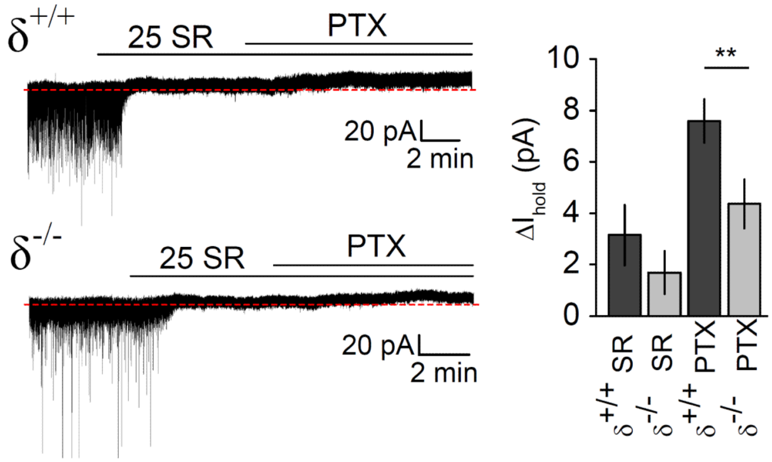

Figure 2.

δ subunit-containing $GABA_A$ receptors are involved in generating picrotoxin-sensitive tonic currents in DGCs. The effects of SR95531 (25 μM) and picrotoxin (PTX, 100 μM) on holding currents recorded in wild-type (*top trace*) and knockout mice lacking δ subunit-containing $GABA_A$Rs (*bottom trace*). Picrotoxin-sensitive tonic currents are reduced in the knockout mice compared to the wild-type littermate controls (n = 9 and n = 10 for the $\delta^{+/+}$ and $\delta^{-/-}$ mice respectively, ** $P < 0.01$, Student's unpaired t-test). Histogram shows mean values ± SEM.

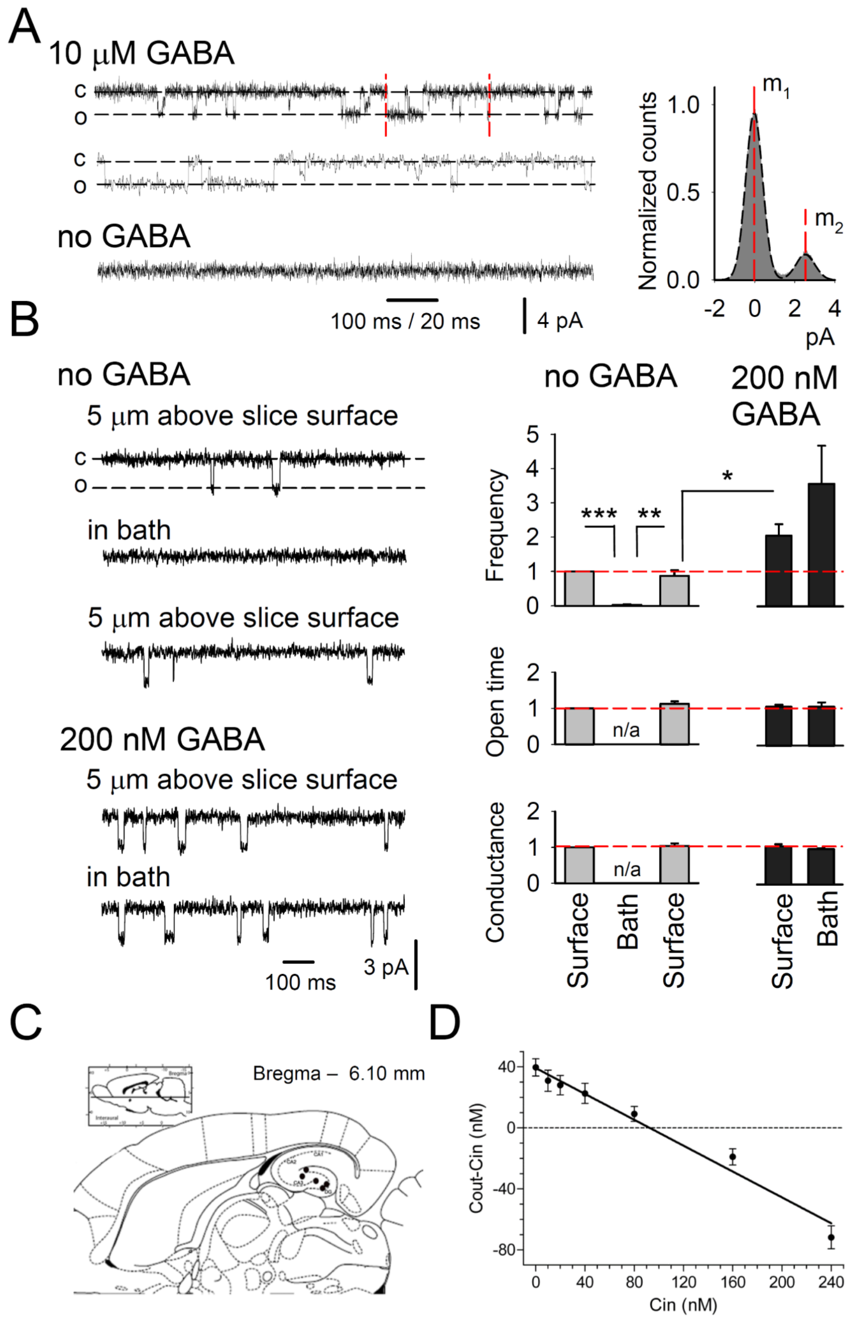

Figure 3.

Low extracellular GABA concentration *in vitro* and *in vivo*. ***A***, "Sniffer" patches - outside-out patches from DGCs display $GABA_AR$ channel openings in the presence of GABA. Representative traces are shown on the left panel. *Top*: Application of 10 μM GABA to the outside-out patches induces single-channel openings. *Middle*: A blow-up of the section of the top trace marked by the vertical dashed lines. *Bottom*: a trace illustrating no channel activity in the absence of GABA in perfusion solution. *Right panel*: normalized all-points amplitude histogram from the experiment with 10 μM GABA application. m_1 and m_2 - mean current amplitude values. Horizontal lines indicate mean amplitude values of an open (O) and closed (C) state. ***B***, The frequency of channel opening in the "sniffer" patch close to

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slice surface (5 μm above DGC layer) and in the bath (300 μm above the slice surface) with and without 200 nM GABA in the perfusion solution (sample traces from the same patch at different conditions are shown *on the left*). Bar charts show (*from top to bottom on the right*) normalized values of channel opening frequency, open time and conductance ± SEM (n = 5; * P < 0.05; ** P < 0.01; *** P < 0.001, paired Student's t-test). ***C***, Estimation of $[GABA]_e$ in the rat hippocampus was performed using zero-net-flux microdialysis. Locations of the microdialysis probes in the hippocampus from 5 rats are shown by the black circles on the schematic of a horizontal section of rat brain (Paxinos and Watson, 2007). ***D***, After collection of six baseline samples, the microdialysis probe was perfused with increasing concentrations of GABA (C_{in}; 10-240 nM) by reverse dialysis. The baseline condition represents C_{in} = 0. The concentration of GABA in the collected samples (C_{out}; nM) was determined by HPLC with electrochemical detection. The net loss or gain of GABA in the dialysate (C_{out} - C_{in}; nM) was calculated and plotted against the concentration of GABA in the perfusion fluid (C_{in}). The intercept with the dashed line (y = 0) is the concentration of GABA at which $C_{out} = C_{in}$ represents an estimate of the $[GABA]_e$ (n=5).

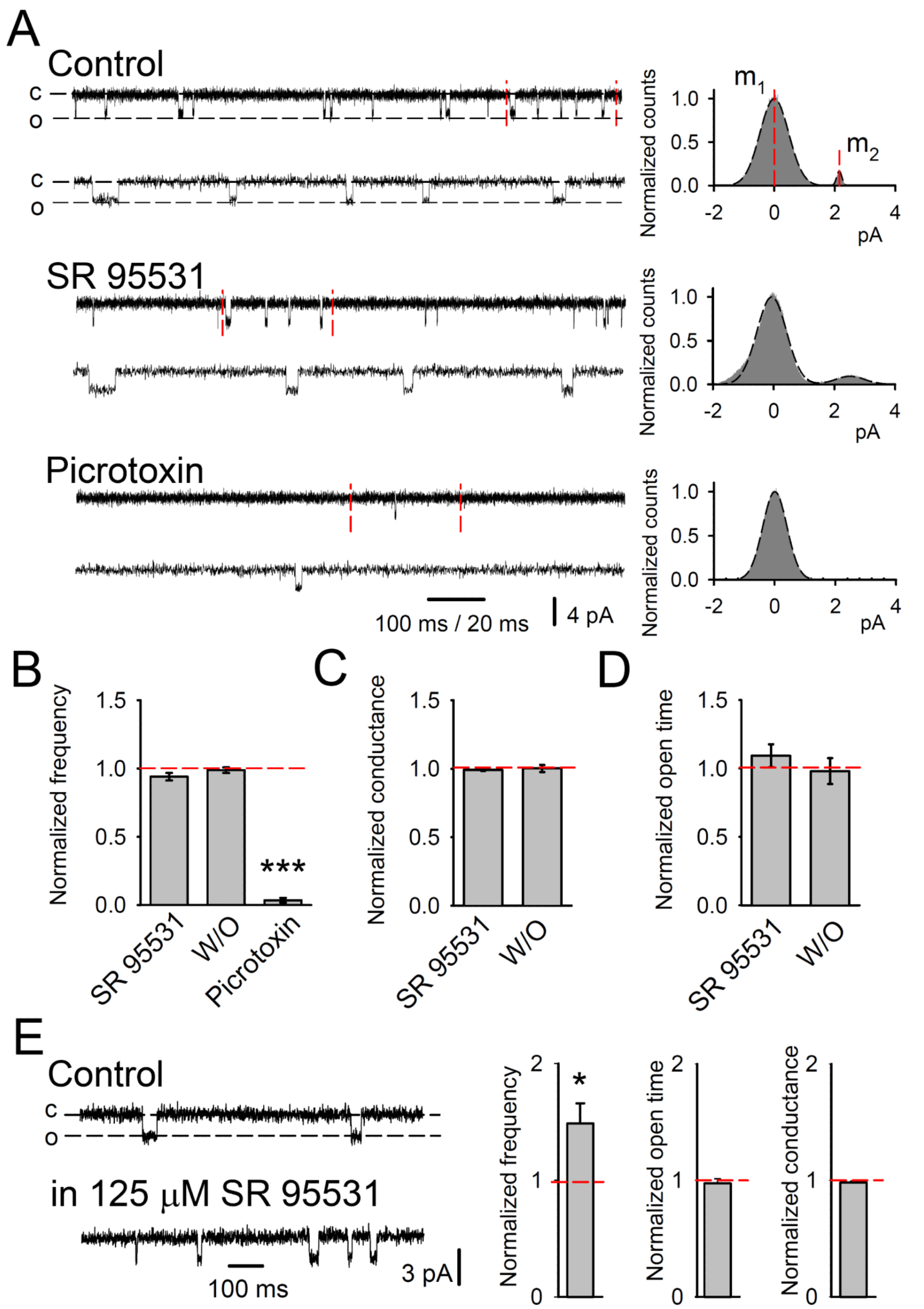

Figure 4.
Spontaneous GABA$_A$R channel openings in nucleated patches. ***A**, Left*: representative traces illustrating single-channel openings, *top* to *bottom*: control, SR95531 (25 μM), picrotoxin (20 μM). Lower traces are expanded segments indicated by vertical dashed lines on the upper traces. *Right*: normalized all-points amplitude histograms. m_1 and m_2, - mean current amplitude values. Channel opening frequency (***B***), conductance (***C***) and average open time (***D***) normalized to control values (n = 5 nucleated patches). ***E***, Application of 125 μM SR95531 increases the frequency of spontaneous channel openings. *Left*: sample traces; *right*: spontaneous channel opening frequency, open time and conductance normalized to

control values (n = 5). Horizontal dashed lines indicate an open (O) and closed (C) state. * $P < 0.05$; *** $P < 0.001$, paired t-test, W/O – washout.

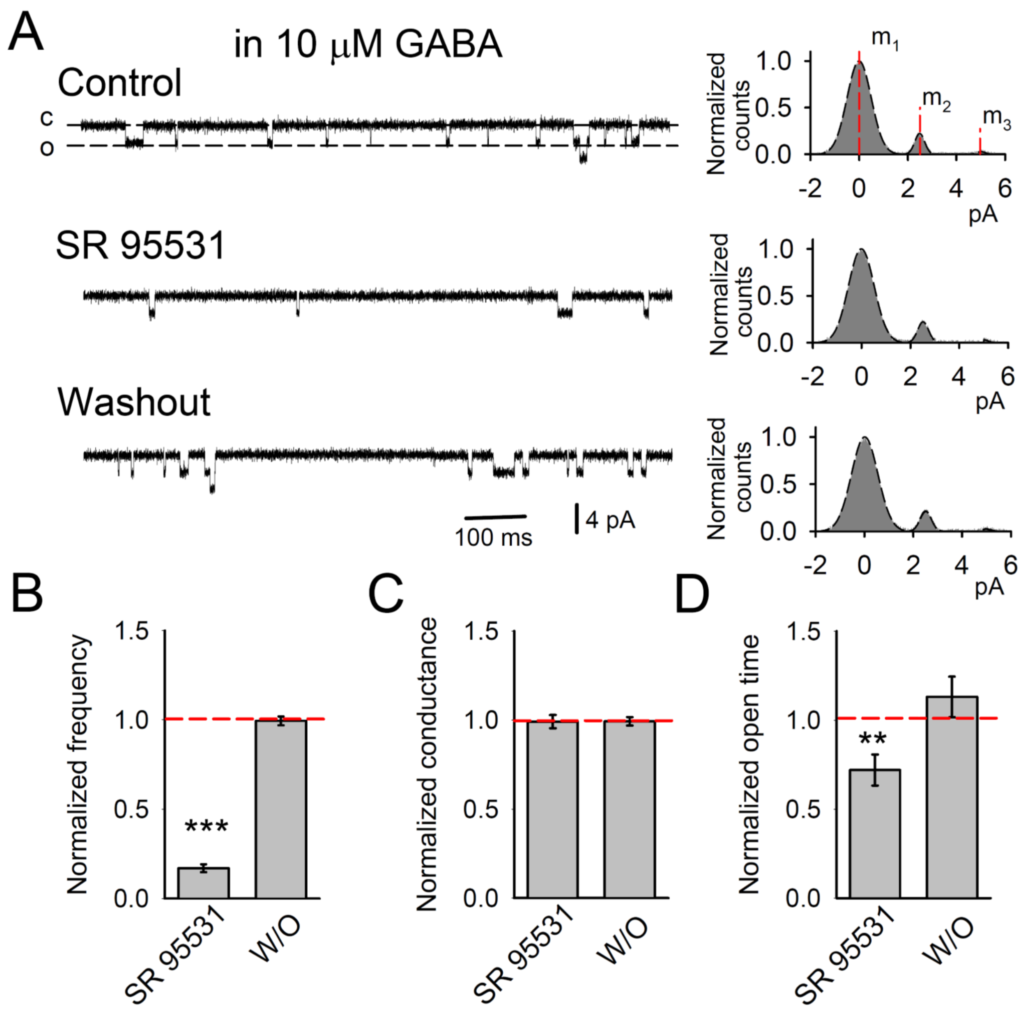

Figure 5.

Application of GABA to nucleated patches from dentate granule cells induces SR95531-sensitive channel openings. ***A**, Left panel*: Representative traces illustrating single-channel openings at consecutive stages of the experiment carried out in the presence of 10 μM of GABA. From *top* to *bottom*: control, SR95531 (25 μM), washout of SR95531. Horizontal dashed lines indicate mean amplitude values of an open (O) and closed (C) state. *Right panel*: normalized all-points amplitude histograms. m_1, m_2, m_3 - mean current amplitude values. Normalized average opening frequency (***B***), conductance (***C***) and open time (***D***) of channel openings induced by GABA application (n = 5 nucleated patches). ** $P < 0.01$, *** $P < 0.001$, paired Student's t-test; W/O – washout.

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Table 1

The effect of different GABA antagonists in control conditions with no GABA added.

Antagonist	**GABA tonic current (ΔI_{hold}, pA)**
PTX (100 μM)	11.9 ± 1.5, n = 9, P = 0.00004 compared to control
Pentylenetetrazol (1.5 mM)	10.6 ± 3.6, n = 5, P = 0.04 compared to control
Bicuculline (10 μM)	5.9 ± 0.6, n = 6, P = 0.0002 compared to control
SR95531 (125 μM)	−6.7 ± 1.6, n = 5, P = 0.01 compared to control
PTX (100 μM) in SR95531 (125 μM)	18.9 ± 3.4, n = 5, P = 0.005 compared to SR95531 (125 μM)